SUMMARY OF THE INVENTION:

Calf-chymosin gene is isolated preferably from the fourth stomach of milk fed calf tissues. Recombinant calf-chymosin is produced by cloning chymosin gene with bacterial expression vector PET21b and is transformed to E-coli strain. This E-coli strain containing recombinant calf-chymosin gene is fermented under suitable conditions preferably in a culture medium developed by us. This medium contains the following

Peptone – 12g/l

Yeast Extract – 24g/l

Sodium chloride – 10g/l

Prochymosin produced during fermentation is subjected to denaturation by increasing the pH of the medium to 10-11. The suspension then diluted and the pH reduced to about 8 for effective renaturation of the protein. The prochymosin thus obtained is then acidified for activation and is further processed.

This invention relates to a process for producing recombinant calf-chymosin which comprises the steps of isolating calf-chymosin gene, cloning the same in bacterial expression vector PET 21b, transforming said cloned vector into cells of E-coli, fermenting said E-coli strains to produce pro-chymosin, converting said prochymosin to chymosin and subsequently recovering the recombinant calf chymosin.

[REPLACEMENT SHEET]

This invention also includes recombinant calf-chymosin having the following amino acid sequence:

```
   MetAlaSerIle ThrArgIle ProLeuTyr LysGlyLysSer LeuArgLys AlaLeuLys
 1 ATGGCTAGCA TCACTAGGAT CCCTCTGTAC AAAGGCAAGT CTCTGAGGAA GGCGCTGAAG
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   CTCGTACCCG AAGACCTCCT GAAGGACGTC TTTGTCGTCA TACCGTAGTC GTCGTTCATG
   SerGlyPheGly GluValAla SerValPro LeuThrAsnTyr LeuAspSer GlnTyrPhe
121 TCCGGCTTCG GGGAGGTGGC CAGCGTGCCC CTGACCAACT ACCTGGATAG TCAGTACTTT
   AGGCCGAAGC CCCTCCACCG GTCGCACGGG GACTGGTTGA TGGACCTATC AGTCATGAAA
   GlyLysIleTyr LeuGlyThr ProProGln GluPheThrVal LeuPheAsp ThrGlySer
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   CCCTTCTAGA TGGAGCCCTG GGGCGGGGTC CTCAAGTGGC ACGACAAACT GTGACCGAGG
   SerAspPheTrp ValProSer IleTyrCys LysSerAsnAla CysLysAsn HisGlnArg
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   AGACTGAAGA CCCATGGGAG ATAGATGACG TTCTCGTTAC GGACGTTTTT GGTGGTCGCG
   PheAspProArg LysSerSer ThrPheGln AsnLeuGlyLys ProLeuSer IleHisTyr
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   AAGCTGGGCT CTTTCAGCAG GTGGAAGGTC TTGGACCCGT TCGGGGACAG ATAGGTGATG
   GlyThrGlyLys MetGlnGly IleLeuGly TyrAspThrVal ThrValSer AsnIleVal
361 GGGACAGGCA AGATGCAGGG GATCCTGGGC TATGACACCG TCACTGTCTC CAACATTGTG
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   CTGTAGGTCG TCTGTCATCA GGACTCGTGG GTCCTCGGGC CCCTGCAGAA GTGGATACGG
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   CAGTGGTAGT CGCCACACCA ACACCGGACA CTCCCACCGA CAGTCCGGTA GGACCTGTGC
   GlyThrSerLys LeuValGly ProSerSer AspIleLeuAsn IleGlnGln AlaIleGly
781 GGCACCTCCA AGCTGGTCGG GCCCAGCAGC GACATCCTCA ACATCCAGCA GGCCATTGGA
   CCGTGGAGGT TCGACCAGCC CGGGTCGTCG CTGTAGGAGT TGTAGGTCGT CCGGTAACCT
   AlaThrGlnAsn GlnTyrAsp GluPheAsp IleAspCysAsp AsnLeuSer TyrMetPro
841 GCCACACAGA ACCAGTACGA TGAGTTTGAC ATCGACTGCG ACAACCTGAG CTACATGCCC
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```


[REPLACEMENT SHEET]

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1021 CTGGGGGATG TTTTCATCCG AGAGTATTAC AGCGTCTTTG ACAGGGCCAA CAACCTCGTG
     GACCCCCTAC AAAAGTAGGC TCTCATAATG TCGCAGAAAC TGTCCCGGTT GTTGGAGCAC
     GlyLeuAlaLys AlaIle***
1081 GGGCTGGCCA AAGCCATCTG A
     CCCGACCGGT TTCGGTAGAC T
```

In the above sequence, amino acids shown in red indicate sequence variation of chymosin gene of our invention compared to the reported and published sequence.

[REPLACEMENT SHEET]

PCR amplification of prepro chymosin was performed using the 50ng of 1st strand cDNA with a reverse primer (5'-TGT GGG GAC AGT GAG GTT CTT GGT C-3'), and a forward primer (5'-ATG AGG TGT CTC GTG GTG CTA CTT-3') in a thermal cycler programmed as (step 1: 95-5'; step 2: 94-30sec.; step 3: 54-30sec; step 4: 72-1min; step 5: go to step 2 34 times; step 6: 72-7min; step 7: end). The PCT reaction when analyzed on 1.0% agarose gel showed an amplified band of 1.2kb. The 1.2kb fragment was cut with a sterile blade and the gel slice was dissolved in 500µl of Tris saturated phenol was added and left in liquid nitrogen for a few min. The microcentrifuge tube was allowed to come to room temperature and centrifuged for 5min at 12,000rpm, 4°C. The upper aqueous phase was extracted with phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and DNA was precipitated with 1/10th volume sodium acetate and 2.5 volume ethanol at –70°C for 1 h. DNA was precipitated at 15,000rpm for 15 min. The pellet was dried and dissolved in sterile distilled water. This eluted 1.2kb fragment was ligated at SmaI site of pBSSK+ plasmid, which was then transformed in to TOP10 cells of E.coli. The recombinant clones were selected (blue white screening) and checked with restriction digestion analysis of the plasmids. Recombinant plasmid was taken as a template and a PCR was performed using a forward primer (5'-GAT ATA CAT ATG GCT AGC ATC ACT AGG ATC CCT CTG TAC-3') and reverse primer (5'-GCA GTA AGC TTG ACA GTG AGG TTC CTT GGT CAG CG-3') containing Nde 1 and Hind III sites. An amplified band of 1098bp was observed when the PCR product was analyzed on 1.0% agarose gel. This amplified fragment of 1098bp was eluted from the gel and ligated in pET21b expression vector at Nde 1 and Hind III sites and transformed in to BL21 cells of E.coli for the expression of the chymosin gene.

[REPLACEMENT SHEET]

CLAIMS:

1. A process for producing recombinant calf-chymosin which comprises the steps of isolating calf-chymosin gene, cloning the same in bacterial expression vector PET21b, transforming said cloned vector into cells of E.coli, fermenting said E.coli strains to produce pro-chymosin, converting said pro-chymosin to chymosin and subsequently recovering the recombinant calf-chymosin.

2. The process as claimed in claim 1, wherein calf-chymosin gene is obtained by isolating RNA from the fourth stomach of calf tissue, synthesising a first strand of cDNA therefrom by treating the same with a reverse primer such as 5'-TGT GGG GAG AGT GAG GTT CTT GGT C-3' and then with a forward primer such as 5'-ATG AGG TGT CTC GTG GTG CTA CTT 3 and with a reverse primer such as 5'TGT GGT GAC AGT GAG GTT CTT GGT C-3'.

3. The process as claimed in claims 1 and 2 wherein said C DNA is ligated at small site of pBSSK+ plasmid and then transformed into TOP 10 cells of E-coli.

4. The process as claimed in claim 3 wherein said recombinant clones were identified and treated with a forward primer such as 5'-GAT ATA CAT ATG GCT AGC ATC ACT AGG ATC CCT CTG TAC 3' and reverse primer such as 5' GCA GTA AGC TTG ACA GTG TTC CTT GGT CAG CG-3' containing Nde I and Hind III sites to obtain an amplified fragment.

5. The process as claimed in claim 4 wherein said amplified fragment is transformed into cells of E.coli for expressing said chymosin gene.

{REPLACEMENT SHEET}

6. The process as claimed in any of the preceding claims wherein said E.coli cells containing recombinant calf chymosin gene is fermented in a medium containing 12g/L peptone, 24g/L of yeast extract and 10g/L of sodium chloride in the presence of supplements for fermentation and the suspended cells produced on completion of fermentation is lysed, chilled and pH adjusted to 8 before incubating at room temperature and the supernatent containing prochymosin is separated.

7. The process as claimed in claim 6, wherein the pH of said prochymosin containing supernatent is adjusted to 2 at room temperature and further incubated for about 6 hrs with gentle stirring and filtered.

8. The process as claimed in claim 7 wherein the pH of said filtrate is adjusted to about 5 and further incubated, filtered and treated with a solution containing sodium benzoate and thereafter a solution containing and sodium chloride to activate prochymosin to chymosin.

9. The process as claimed in claim 8 wherein the filtrate obtained after the addition of sodium benzoate solution is treated with a solution of sodium chloride under stirring and cooking, and the precipitate suspended in a chilled solution of 0.2M glycine with 0.001M EDTA and thereafter treated with 0.23% solution of sodium benzoate and stored under cooling.

10. The process as claimed in claim 9 wherein said chymosin obtained is formulated with 10% of sodium chloride and 0.2% of Trehalose.

REPLACEMENT SHEET

11. Recombinant calf-chymosin having the following amino acid sequence:

```
      MetAlaSerIle ThrArgIle ProLeuTyr LysGlyLysSer LeuArgLys AlaLeuLys
    1 ATGGCTAGCA TCACTAGGAT CCCTCTGTAC AAAGGCAAGT CTCTGAGGAA GGCGCTGAAG
      TACCGATCGT AGTGATCCTA GGGAGACATG TTTCCGTTCA GAGACTCCTT CCGCGACTTC
      GluHisGlyLeu LeuGluAsp PheLeuGln LysGlnGlnTyr GlyIleSer SerLysTyr
   61 GAGCATGGGC TTCTGGAGGA CTTCCTGCAG AAACAGCAGT ATGGCATCAG CAGCAAGTAC
      CTCGTACCCG AAGACCTCCT GAAGGACGTC TTTGTCGTCA TACCGTAGTC GTCGTTCATG
      SerGlyPheGly GluValAla SerValPro LeuThrAsnTyr LeuAspSer GlnTyrPhe
  121 TCCGGCTTCG GGGAGGTGGC CAGCGTGCCC CTGACCAACT ACCTGGATAG TCAGTACTTT
      AGGCCGAAGC CCCTCCACCG GTCGCACGGG GACTGGTTGA TGGACCTATC AGTCATGAAA
      GlyLysIleTyr LeuGlyThr ProProGln GluPheThrVal LeuPheAsp ThrGlySer
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[REPLACEMENT SHEET]

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1021 CTGGGGGATG TTTTCATCCG AGAGTATTAC AGCGTCTTTG ACAGGGCCAA CAACCTCGTG
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1081 GGGCTGGCCA AAGCCATCTG A
     CCCGACCGGT TTCGGTAGAC T
```

13. Recombinant calf-chymosin when produced by a process according to any of the preceding claims.

{REPLACEMENT SHEET}

ABSTRACT

Calf-chymosin containing 1098 p coding for 366 amino acids was isolated and cloned into a bacterial expression vector pET 21b. This strain is fermented and the protein is precipitated by addition of alkali. Active form is recovered by renaturation and purified under low pH.

[R&PLACEMENT SHEET]

SEQUENCE LISTING

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Sudershan Biotech Limited

<120> Recombinant Calf Chymosin and a process for producing the same

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35 40 45

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[REPLACEMENT SHEET]

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Ile Leu Asp Thr Gly Thr Ser Lys Leu Val Gly Pro Ser Ser Asp Ile
            260                 265                 270

Leu Asn Ile Gln Gln Ala Ile Gly Ala Thr Gln Asn Gln Tyr Asp Glu
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[REPLACEMENT SHEET]

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[REPLACEMENT SHEET]